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UPPFINNINGENS BENÄMNING: A NEW PROBIOTIC COMPOSITION

The invention relates to a new probiotic composition. More precisely, the invention refers to a probiotic composition comprising at least two lactic acid bacterial strains having at least two important properties for the maintenance of the intestinal microbial ecosystem, for the prevention and treatment of gastrointestinal disturbances, and for colonizing gastrointestinal tracts.

The enteric flora comprises approximately 95% of the total number of cells in the human body. The importance of the intestinal microflora and, more specifically its composition, in physiological as well as pathophysiological processes in the human gastrointestinal tract has become more and more evident.

Health effects related to changes in the intestinal microflora have been attributed to viable microorganisms (bacteria or yeast) that have a beneficial effect on the health of the host. The presence of lactic acid bacteria has been found to be important for the maintenance of the intestinal microbial ecosystem. These microorganisms, since long called probiotics, are commonly defined as viable that exhibit a beneficial effect on the health of the host when they are ingested. Thus, a probiotic can be defined as a viable monoculture or a mixed culture of microorganisms, which affects the host by improving the properties of indigenous microflora in the gastrointestinal tract. Presently, a number of commercial products are available for the prevention and treatment of multiple gastrointestinal disturbances.

In EP 1 020 123 A1 beverages in combination with a mixture of lyophilized live lactic bacteria in a non-milk matrix are described. The mixture of lyophilized live

lactic bacteria comprises at least three of *Brevibacterium breve, B. infantis, B. longum, B. bifidum, Lactobacillus acidophilus, L. bulgaricus, L. casei, L. plantarum, Streptococcus thermophilus* and *S. faecium*. The beverages are intended to supplement and balance the intestinal flora as well as supply other beneficial supplements, such as vitamins and antioxidants, to the consumer.

However, the literature contains many conflicting observations for their proposed benefits, and the corresponding mechanism of action is many times undefined.

Possibly successful probiotic strains have been traditionally incorporated into fermented milk products. In the case of novel microorganisms and modified organisms the question of their safety and the risk to benefit ratio have to be assessed. Lactic acid bacteria in foods have a long history of safe use.

The last few years these organisms have been included in functional foods and health-related products. The definition for probiotics has gradually changed with increasing understanding of the mechanisms by which they influence human health. While the health claims are generally accepted by both scientists and consumers, the underlying molecular mechanisms of many of the claimed probiotic properties still remain controversial.

Lactic acid bacteria have successfully been isolated and identified, which exhibit beneficial probiotic traits. These characteristics include the demonstration of bile tolerance; acid resistance; adherence to host epithelial tissue; and in vitro antagonism of potentially pathogenic microorganisms or those which have been implicated in promoting inflammation.

On the market, there exist several milk and fruit based products containing lactic acid bacteria. The specific interactions with the gastrointestinal tract are often sparsely described, and the doses poorly defined.

The probiotic microorganisms must be able to be manufactured under industrial conditions. Furthermore, they have to survive and retain their functionality during storage, and also in the foods into which they are incorporated without producing negative effects. Studies have shown low viability of probiotics in market preparations.

For the administration of lactic acid bacteria as probiotics in humans or animals, they should be adapted to the specific conditions of the gastrointestinal tract. However, up to now there exist poor evidence of a successful implanting of a given strain into the dominant flora of a healthy individual. Such an implantation can succeed only at the moment of birth or when the probiotic organism is administered to a patient having an extremely unbalanced intestinal microflora, for example after prolonged antibiotic treatment.

The purpose of the invention is to avoid the above-mentioned drawbacks of the known technique by producing a probiotic composition with optimal capacity to survive and colonize the gastrointestinal tracts of humans and animals.

In order to achieve this purpose the method according to the invention has obtained the characterizing features of claim 1.

Two or more lactic acid bacteria strains of different species have jointly properties that should be beneficial for human intake while simulating the effect of an implanted flora. By having at least two properties together, which define well-established criteria for gastrointestinal survival and/or colonization, the potential health benefits and influence on the gut flora is increased. According to the invention new probiotic strains of active beneficial organisms are provided with specific favourable functional characteristics.

Strains of the inventive composition were isolated from the human large intestinal mucosa of deceased persons

who died from other causes than diseases in the GI tract, and whom had not been treated by antibiotics during at least two months prior to death (F strains and 2362). The other strains were isolated from fermented rye.

The strains were typed to the species level by API 50CH as well as ribotyping (the Swedish Institute for Food and Biotechnology).

Representative strains to be used in the probiotic composition according to the invention are *Lactobacillus plantarum* F5, *Lactobacillus plantarum* F26, *Lactobacillus plantarum* 2592, *Lactobacillus paracasei* (*paracasei*) F19, *Pediococcus penosaceus* 16:1, *Lactobacillus plantarum* 50:1, and *Leuconostoc mesenteroides* 77:1.

Bacterial strains were deposited on June 19, 2001 pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure with the Belgian Coordinated Collection of Microorganisms (BCCM), Gent, Belgium, under Accession No. LMG P-20604 for *Lactobacillus plantarum* F5, Accession No. LMG P-20605 for *Lactobacillus plantarum* F26, Accession No. LMG P-20606 for *Lactobacillus plantarum* 2592, Accession No. LMG P-20607 for *Leuconostoc mesentorides* 77:1, and Accession No. LMG P-20608 for *Pediococcus penosaceus* 16:1.

Lactobacillus plantarum 50:1 was deposited with the Belgian Coordinated Collection of Microorganisms, on June 21, 2001, where it obtained the Accession No. P-20609.

Lactobacillus paracasei (*paracasei*) F19 has earlier been deposited with the Belgian Coordinated Collection of Microorganisms, where it obtained the Accession No. LMG P-17806.

All strains were grown aerobically on MRS agar at 37°C for 24 hrs. In a further experiment, growth at different temperatures was determined. All strains could multiply at temperatures from +4°C to 40 or 45°C. All strains pro-

duced protease(s) as determined by skim milk agar assay (24 hrs, aerobically, 37°C). No strain produced nitrite or nitrate.

All strains could utilize prebiotics, such as inulin and amylopectin, but not β-glucan as determined by growth on YNB medium with these fibers as a sole carbon source. Since they ferment fibers, they should exert a beneficial effect in the colonic flora. An enhanced fiber degradation also increases the crude fibre digestibility in ruminants.

In a composition according to the invention a mixture of cocci and bacilli should be optimal since cocci and bacilli have different generation times, bacilli proliferating much faster than cocci. This is accomplished by preferably including at least one lactic acid bacillus strain and at least one lactic acid coccus strain in the composition.

In order promote growth of the lactic acid bacterial strains and succeed it the colonization of the epithelium of the gastrointestinal tract of a host as well as exerting a resistance to infectious diseases a composition according to the invention should have an intestinal survival property, an intestinal binding property, an infection protecting property, and a fiber fermenting property.

An important intestinal survival property is the ability to grow in the presence of bile. All the claimed strains are able to grow in the presence of 20% bile (human and porcine) and then retain their bile-tolerance after the selective pressure has been removed and reapplied.

All the strains also survive when they are subjected to an acidity of pH 2.0-3.0. Furthermore, this acid resistance remains with the addition of 0.3 % pepsin (24 hrs at 37°C) in three of them (*L. plantarum* F5, *L. plantarum* F26, and *L. plantarum* 50:1) as depicted in Table 1 below.

Table 1

Strain	pH	pepsin
L. plantarum F5	2.0	+
L. paracasei (*parac.*) F19	2.5	
L. plantarum F26	2.0	+
L. plantarum 2592	2.5	
P. pentosaceus 16:1	2.5	
L. plantarum 50:1	2.0	+
L. mesenteroides 77:1	3.0	

Thus, the acid and bile tolerant strains possess growth advantages over that of the parents under stress conditions.

BalbC mice were fed inventive lactic acid bacteria intragastrically. The strains used were *L. paracasei* (*paracasei*) F19, *L. plantarum* 2592, *P. pentosaceus* 16:1, and *L. mesenteroides* 77:1. The excretion of the four strains given was obtained by culturing. Excretion of these could be during 6 weeks after the administration, which indicates colonization of the gastrointestinal tract of the host.

The acid response of these strains when exposed to sublethal adaptive acid conditions (pH 5.0 for 60 min) was found to confer a significant level of protection against subsequent exposure to lethal pH (pH 3.0) as well as to different environmental stresses (oxidative, ethanol and freezing).

An acid tolerant response developed during adaptation at pH 5.0 affected the cell survival against environmental stresses. Adapted cultures developed tolerance to ethanol (20%), freezing (-20°C), and oxidative challenge (10 mM H_2O_2) but not to heating (60°C) and osmotic shock (3 M NaCl). The presence of chloramphenicol during the adaptation step partially inhibited the cell resistance. This ad-

aptation was found to be dependent on a *de novo* protein synthesis.

An exposure to acid stress at pH 5.0 for 1 h caused the induction of nine new proteins with molecular weights (MW) from 10.1 to 68.1 kDa as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis, whereby the overexpression of the proteins also could be established. All other proteins with basic or neutral characteristics were repressed.

The proteins induced during this acid tolerance response have to be active since the incorporation of amino acid analogues inhibited the response.

Several of the induced and over-expressed proteins were also found to cross-react with earlier described heat shock proteins. Proteins of molecular weight 10, 24 and 43 kDa crossreacted with cochaperons GroES, GrpE and DnaJ, respectively. Less over-expressed proteins of molecular weight70 and 55 kDa cross-reacted with GrpE and GroES, respectively.

Thus, the survival under acid stress conditions was found to be linked to the expression of an adaptive stress response. Such a response, characterized by the transient induction of specific proteins and physiological changes, enhance their ability to withstand harsh environmental conditions. The continued protein synthesis of specific proteins induced by the claimed strains in an acid environment, like in the gastric stomach, would then increase the stability of preexisting proteins.

Lactobacillus plantarum 2592 produces large amounts of a characteristic protein having a molecular weight of 19-kDa.

A further infection protecting property of the inventive strains is their antioxidant properties, whereby the action of free radicals, by products of inflammation, are counteracted.

The strains produced antioxidants as measured by a spectrophotometric assay (Total Antioxidant kit, product no. NX 2332 from Randox, San Diego, CA, USA.) in lysates of lactic acid bacterial strains. It was shown that anti-oxidants are produced which are effective against free radicals and terminate oxidative chain reactions. All the strains produced antioxidants in amounts from 2.7 to 8.9 mg protein per lit. The strains *P. pentosaceus* 16:1 and *L. plantarum* F26 produced the largest amounts of antioxidants immediately followed by *L. paracasei* (*paracasei*) F19.

Diabetic mice were given these strains in the same dose daily for 12 days. The mice were fed 10^{10} cells of four strains (*L. paracasei* (*paracasei*) F19, *L. plantarum* 2592, *P. pentosaceus* 16:1, and *L. mesenteroides* 77:1) twice daily of these strains intragastrically for 12 days. The cholesterol levels of the animals were not decreased. The safety of the strains was confirmed by taking blood cultures of the mice at the end of the study, which showed no growth.

Likewise, 52 healthy persons (14-87 years of age) consumed 10^{10} bacteria of 4 of these strains daily for 3 months without adverse effects.

Preferably, the infection protecting property of the inventive composition is an immunopotentiating effect, whereby a positive immune response is obtained. The strains were found to have different abilities of transcribing NF-kappa B to the cell nucleus as determined by a dot blot (Wilson L, et al., Gastroenterol 1999; 117:106-114; and Splecker M, et al., J Immunol 2000;15 (March):3316-22). The induction of NF-kappa B results in a cytokin response, which either is pro-inflammatory or anti-inflammatory.

The *Lactobacillus* strains, particularly *L. paracasei* (*paracasei*) F19 but not the cocci, transcribed NF-kappa B in the macrophage cell line U973, resulting in the syn-

thesis of the interleukins IL-1β and IL-8. The cytokines induced were of the pro-inflammatory type.

A further important aspect of the composition according to the invention is that probiotic strains shall an intestinal binding property in order to be able to developing their functional properties. One such property is that they shall exhibit high adhesion to intestinal tracts. To act as a probiotic, a lactic acid bacterial strain must be able to colonize the intestinal mucus layer.

The claimed strains were also obtained by screening for binding of porcine mucin (type II, Sigma Chem Co, St Louis, CO, USA)) in an ELISA method (Tuomola EM, *et al.*, FEMS Immunology and Med Microbiol 26(1999) 137-142). The results are shown in Table 2 below. The results were compared with a commercial strain *Lactobacillus rahmnosus* GG (Valio).

Table 2

Strain	Mucin binding	SAT
L. plantarum F5	0.557	1M
L. paracasei (parac) F19	0.515	0.1M
L. plantarum F26	0.582	1M
L. plantarum 2592	0.707	1M
P. pentosaceus 16:1	1.052	<0.1M
L. plantarum 50:1	0.883	2M
L. mesenteroides 77:1	1.265	<0.1M
L. rahmnosus GG	0.2	4M

Apart from exhibiting mucin binding, the probiotic strains in the inventive composition should be able to express cell surface hydrophobicity in human gastrointestinal tract. The cell surface hydrophobicity was measured by the Salt Aggregation Test (SAT) (Rozgoynyi F, *et al.*, FEMS Microbiol Lett 20(1985) 131-138). In Table 1 below, a comparison is shown for the adhesiveness of vibronectin (Vn),

vibronectin at pH 3.5, fetuin (Ft), asialofetuin, and asialofetuin at pH 3.5.

Table 3

Strain	Vn	Vn pH3.5	Ft*	asialo-Ft	asialo-Ft pH3.5
L. plantarum F5	++	+++	-	+	+++
L. paracasei F19	+++	+++	+++	+++	+++
L. plantarum F26	+	+++	+	+++	+
L. plantarum 2592	-	+	+	+++	-
P. pentosaceus 16:1	+++	+	-	+	-
L. plantarum 50:1	+++	+	-	++	+
L. mesenteroides 77:1	-	++	-	+	+

* denotes identical results at pH 7 and pH 3.5

Adhered microorganisms were found to be tightly bound to the immobilized mucus. Five strains expressed a pronounced cell surface hydrophobicity and the other three moderate cell surface hydrophobicity. No correlation was observed between cell surface hydrophobicity and the adhesive ability of the strains.

The strains were also tested for binding to bovine submaxillary glands and porcine type II mucin (Sigma) in a particle agglutination assay (PAA) (Paulsson M, *et al.*, J Clin Microbiol 30(1992) 2006-20012). Generally, expression of binding to bovine mucus was found to be stronger.

Since many of the cells in tissues of multicellular organisms are embedded in an extracellular matrix consisting of secreted proteins and polysaccharides, the inventive strains of lactic acid bacteria were examined with reference to their interaction with gastrointestinal extracellular matrix protein. The binding of extracellular matrix proteins and glucosaminoglycans was studied in the above mentioned immobilized form (PAA assay). All the strains expressed binding to collagen type I and III,

fibronectin, fibrinogen, and heparin, also at pH 3.5. Strains *L. paracasei (paracasei)* F19, *L. plantarum* F26, and *L. plantarum* 2592 expressed binding of fetuin, also at pH 3.5.

Furthermore, all strains, except *L. plantarum* 2592 and *L. mesenteroides* 77:1, expressed binding to vitronectin. However, at pH 3.5, these strains expressed a weak binding.

A further important intestinal survival property of the lactic acid bacterial strains in the probiotic composition according to the invention is to have an infection protecting property.

Heliobacter pylori is the causative agent of acute and chronic gastritis, one of the most prevalent infections world-wide which may proceed to atrophic gastritis, adenocarcinoma and MALT lymphoma.

The lactobacilli of the present invention to inhibit growth of 10 strains of *H. pylori* due to the concentration of lactic acid and the pH *in vitro*. The inhibitory effect was lost when the pH was adjusted to 6.0. Further analyses showed that L-lactic acid but not D-lactic acid or acetic acid inhibited growth at concentrations of 60 to 100 mM. No relation between CagA phenotype of *H. pylori* and tolerance to lactic acid was observed. The inhibition was found to be strain-specific.

A further infection protecting property is manifested by interactions mediated by bacteriocins. All strains, except *L. plantarum* F5, secrete into a cell-free supernatant a product having antimicrobial activity. The products produced were heat-stable antimicrobial compounds, which were shown to be proteinaceous in nature and, therefore, referred to as a bacteriocins. The bacteriocins exhibited activities against grampositive organisms. Some were active against gramnegative organisms (*H. pylori* strains), and some against yeasts (Candida strains). The

antimicrobial effects were not directly correlated to the production of acid.

Furthermore, *H. pylori* cells were bacillary in their shape and not coccoid, indicating that the observed inhibition was related to a bactericidal effect rather than induction of viable but non-culturable coccoid forms. The bactericidal effect was found to be due to an intracellular 28 kDa protein, which was released after lysis. Proteolytic treatment of this intracellular protein resulted in loss of antibacterial activity. This loss could be abolished by renaturing by means of sodium dodecyl sulphate polyacrylamide gel electrophoresis.

In an established model of *H. pylori* gastritis in mice administration of lactic acid bacillus strains according to the invention resulted in inhibited infection and decreased inflammation. This inhibitory effect appeared to be strain-specific rather than species-specific. The *in vitro* activity of the lactic acid bacterial strains correlated well with activity against *H. pylori*.

Preparations of the probiotic composition according to the invention comprise chilled, frozen, or lyophilized live bacteria of at least 10^{10} CFU/g as a probiotic additive in food or feed.

CLAIMS

1. A probiotic composition comprising at least two lactic acid bacterial strains, characterized in that said at least two lactic acid bacterial strains are able colonize the gastrointestinal tracts of humans and animals and in combination have at least two beneficial properties, which are an intestinal survival property, an intestinal binding property, an infection protecting property, and a fiber fermenting property, said at least two lactic acid bacterial strains being selected from the group comprising *Lactobacillus plantarum* F5 (LMG P-20604), *Lactobacillus plantarum* F26 (LMG P-20605), *Lactobacillus plantarum* 2592 (LMG P-20606), *Pediococcus penosaceus* 16:1 (LMG P-20608), and *Leuconostoc mesentorides* 77:1 (LMG P-20607), *Lactobacillus plantarum* 50:1 (P-20609), and *Lactobacillus paracasei* (*paracasei*) F19 (LMG P-17806).

2. A probiotic composition as in claim 1, characterized in that said lactic acid bacterial strains are viable bacteria of at least 10^{10} CFU/g.

3. A probiotic composition as in claim 1, characterized in that said intestinal survival property is ability to grow in the presence of bile.

4. A probiotic composition as in claim 1, characterized in that said intestinal survival property is ability to survive at low pH.

5. A probiotic composition as in claim 4, characterized in that said ability to survive at low pH is survival at low pH in the presence pepsin.

6. A probiotic composition as in claim 1 and 4, characterized in that said intestinal survival property is ability to produce stress proteins.

7. A probiotic composition as in claim 6, characterized in that said stress proteins cross-react with heat shock proteins.

8. A probiotic composition as in claim 1, c h a r a c t e r i z e d in that said intestinal binding property is ability to bind to mucin.

9. A probiotic composition as in claim 1, c h a r a c t e r i z e d in that said intestinal binding property is ability to bind to extracellular matrix proteins.

10. A probiotic composition as in claim 1, c h a r a c t e r i z e d in that said intestinal binding property is ability to bind to glucosaminoglycans.

11. A probiotic composition as in claim 1, c h a r a c t e r i z e d in that said intestinal binding property is ability to express cell surface hydrophobicity.

12. A probiotic composition as in claim 1, c h a r a c t e r i z e d in that said infection protecting property is ability to produce bacteriocins.

13. A probiotic composition as in claim 12, c h a r a c t e r i z e d in that said bacteriocins have activity against grampositive bacteria.

14. A probiotic composition as in claim 12, c h a r a c t e r i z e d in that said bacteriocins have activity against gramnegative bacteria.

15. A probiotic composition as in claim 12, c h a r a c t e r i z e d in that said bacteriocins have activity against yeast.

16. A probiotic composition as in claim 1, c h a r a c t e r i z e d in that said infection protecting property is ability to produce antioxidants.

17. A probiotic composition as in claim 1, c h a r a c t e r i z e d in that said infection protecting property is ability to induce a pro-inflammatory cytokin response.

18. A probiotic composition as in claim 1, c h a r a c t e r i z e d in that said fiber fermenting property is ability to ferment amylopectin and inulin.

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19. Use of a lactic acid bacterial strain, selected from the group comprising *Lactobacillus plantarum* F5 (LMG P-20604), *Lactobacillus plantarum* F26 (LMG P-20605), *Lactobacillus plantarum* 2592 (LMG P-20606), *Pediococcus penosaceus* 16:1 (LMG P-20608), and *Leuconostoc mesentorides* 77:1 (LMG P-20607), and *Lactobacillus plantarum* 50:1 (P-20609), alone or in combination, as a probiotic additive in food or feed.

ABSTRACT

In a probiotic composition comprising at least two specific lactic acid bacterial strains, the strains are able colonize the gastrointestinal tracts of humans and animals and in combination have at least two beneficial properties.The properties include an intestinal survival property, an intestinal binding property, an infection protecting property, and a fiber fermenting property.